

Bacteriophage Mu DNA Replication *in Vitro**

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An *in vitro* system for bacteriophage Mu DNA replication using lysates on cellophane discs is described. Mu replication was monitored by DNA hybridization. Using a thermoinducible Mu lysogen, 30-50% of all DNA synthesis *in vitro* was Mu-specific.

Mu DNA synthesis is semidiscontinuous. In the presence of the DNA ligase inhibitor NMN, about one-half of the DNA was in Okazaki pieces and one-half in large DNA. The Mu Okazaki pieces hybridized mainly to the Mu light strand; the large DNA hybridized mainly to the Mu heavy strand. Okazaki pieces isolated from uninfected cells also hybridized to 2000-3000 bases of host DNA present in Mu-separated strands. However, the host Okazaki pieces hybridize to both Mu strands symmetrically. Most, if not all, host sequences were represented in mature Mu viral DNA.

The *in vitro* data are most consistent with models in which Mu sequences, oriented randomly in both directions in the host chromosome, have recruited a bacterial replisome which traverses the Mu genome from left to right.

The life cycle of the bacteriophage Mu (1) is unusual in that during vegetative growth, replication is coupled to transposition. If a Mu lysogen is induced, transposition of the viral genome to over a hundred sites in the chromosome takes place in less than 1 h. Mu sequences are then packaged with host sequences at both ends (2, 3). The high transposition frequency makes Mu a specially attractive system for biochemical study.

Two phage genes required for the coupled replication-transposition have been defined (4, 5). The A gene is absolutely required both for the initial integration event and vegetative replication. A second Mu gene, B, is also necessary for Mu DNA replication. The substantial amount of Mu DNA synthesis that takes place during the vegetative cycle suggests that replication may serve as a convenient assay for these gene products. The ability to replicate Mu DNA *in vitro* would provide an essential tool for a biochemical dissection of Mu transposition; many unusual features of Mu replication have already been gleaned from genetic and *in vivo* studies (6-10).

In this report, Mu DNA replication *in vitro* is demonstrated using a film lysate technique (11, 12).

MATERIALS AND METHODS

Bacterial Strains and Plasmids—Strain HB101 is an *Escherichia coli* K12 derivative carrying the following genetic markers: *pro*⁻ *leu*⁻

thi⁻ *lacY*⁻ *strR*⁻ *recA* 13, *r*_k⁻ *m*_k⁻, *endoI* (13). Mu lysogens in HB101 were isolated after infection and selection for Mu-immune colonies. Mu virus stocks were isolated from strain 8709, provided by M. Pato (University of Colorado Medical School), which is a K12 strain lysogenic for Mucts (14), or from HB101::Mucts.

***In Vitro* DNA Synthesis**—The *in vitro* DNA assay method was essentially that of Schaller *et al.* (11) with modified conditions of Olivera *et al.* (12). An HB101::Mucts lysogen carrying the thermoinducible prophage was grown in Penassay broth at 32 °C to an A₆₅₀ of 0.2 and then shifted to 42 °C. After 25 min, [³H]thymidine was added for 5 min, the cells were harvested and spread on cellophane discs. Small 1-cm² discs received 10⁸ cells and large 6-cm² discs received 6 × 10⁸ cells. Lysates of ³H-labeled cells were incubated at 30 °C in reaction mixes containing 10 Ci/μmol of [³²P]dGTP, 20 μM deoxynucleoside triphosphate, 1 mM ATP, 0.04 mg of thymidine/ml, and 1 mg of nicotinamide mononucleotide/ml. For some experiments, NMN was substituted by 0.3 mg of NAD/ml.

DNA synthesis on small discs was terminated and acid-insoluble radioactivity was determined as previously described (12). DNA replication on large discs was terminated with 0.2 ml of 0.1 M Tris·HCl, pH 8.0, 0.005 M EDTA, and 0.5% Sarkosyl (TES¹). 5 μg of RNase A were added and incubation carried out at 37 °C for 15 min followed by the addition of 5 μg of pronase and 15 min of incubation at 37 °C. The volume was increased to 0.5 ml with TES, and DNA was sheared by 5 passages through a 27-gauge needle. DNA was extracted 3 times with phenol saturated with 0.1 M Tris base and 5 mM EDTA, extracted twice with chloroform (1:1 mixtures), and then precipitated by addition of 1/10 volume of 3 M sodium acetate and 2 volumes of 95% ethanol. After incubation at -20 °C for 30 min, the DNA was centrifuged in an Eppendorf centrifuge for 5 min. The supernatant was removed and the precipitate resuspended in 0.2 ml of 20 mM Tris·HCl, pH 8.0, 1 mM EDTA.

Reassociation Reactions—Mu viral DNA and calf thymus DNA, each at concentrations of 350 μg/ml, were denatured by the addition of 1/10 volume of 5 N NaOH. Purified DNA from cellophane discs representing less than 6 μg of DNA in 40 μl was denatured by the addition of 4 μl of 5 N NaOH. Reassociation reactions were initiated by the successive additions to experimental DNA of 7.4 μg of denatured Mu or calf thymus DNA, 300 μl of H₂O, 75 μl of 1 M Na acetate, pH 5.6, and 60 μl 20 × SET (SET is 0.15 M NaCl, 0.03 M Tris·HCl, pH 8.0, 2 mM EDTA). At appropriate times, aliquots of the renaturation mixtures were diluted into S-1 reaction buffer containing 100 mM sodium acetate, pH 4.6, 5 mM zinc sulfate, 5% glycerol, 50 mM sodium chloride, and 10-30 units of S-1 nuclease. Digestions were carried out at 37 °C for 1 h and the reaction mixtures were made 10% (v/v) trichloroacetic acid and filtered on GFC filters. After washing with 25 ml of 5% trichloroacetic acid and 10 ml of 95% ethanol, the filters were dried and the amount of radioactivity determined. Hybridization to separated strands was carried out in 200-μl reaction mixes containing 4 × SET, 0.2% Ficoll 400, 0.2% polyvinylpyrrolidone 40, 0.2% bovine serum albumin, 0.1% sodium dodecyl sulfate, and 0.54 μg of Mu heavy or light strands. After incubations at 65 °C, 75-μl aliquots were diluted into 500 μl of S-1 buffer plus 10 units of S-1 nuclease, and incubation was carried out for 1 h at 37 °C. The mixes were then spotted onto GFC filters which were dried, precipitated with trichloroacetic acid, and counted.

Enzymes—Pronase and ribonuclease A were purchased from Sigma. S-1 nuclease was obtained either from Sigma or Boehringer-Mannheim and yielded identical results from each source. DNA restriction enzymes were from Bethesda Research Corp.

¹ The abbreviation used is: TES, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-amino} ethanesulfonic acid.

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Other Methods—Alkaline sucrose gradients were handled as described by Olivera *et al.* (12). The strands of Mu were separated by the poly(UG) method (15). Phage DNA purified from *E. coli* strain 8709 or HB101 was treated with sodium dodecyl sulfate and phenol extracted 3 times, and DNA was precipitated with ethanol. DNA was resuspended to a concentration of 350 $\mu\text{g}/\text{ml}$ and 1 ml was mixed with 350 μg of poly(UG); the mixture was heated to 95 °C and enough CsCl was added to obtain a density of 1.70. Centrifugation was carried out in a Ti 75 rotor at 50,000 rpm for 24 h. Fractions collected from the bottom of the tube were monitored for absorbance at 260 nm. The heavy fractions and light fractions were pooled and dialyzed against 10 mM Tris, pH 7.6, 1 mM EDTA, 100 mM NaCl. The solution was made 0.1 N in NaOH (by the addition of 1 M NaOH) and incubated at 37 °C for 8 h to remove the bound poly(UG). 1 M Na acetate, pH 5.6, was added to a final pH between 7 and 8, followed by 2 volumes of absolute ethanol; DNA was precipitated by incubation for 2 h at -20 °C, centrifuged and redissolved in 4 \times SET, and allowed to self-hybridize overnight to eliminate contaminating strands in the heavy and light pools.

RESULTS

***In Vitro* Replication Assay**—Studies of bacteriophage Mu DNA replication *in vivo* have revealed a requirement for a number of host genes including *dnaB*, *dnaC*, *dnaE* (16), DNA gyrase,^{2,3} and *himA*,⁴ indicating an extensive dependence on host replication functions. DNA replication on cellophane discs allows host replication forks to continue *in vitro* with characteristics that approximate those *in vivo*. We studied DNA synthesis on cellophane discs using two isogenic *E. coli* strains, one of which is a Mu lysogen. Both strains were harvested after incubation *in vivo* for 30 min at 42 °C, a protocol which induces Mu in the lysogenic strain. For both strains, DNA synthesis continues on the disc for at least 45 min at 30 °C (Fig. 1). Using lysates of HB101::Mucts (which harbors the thermoinducible Mu prophage), DNA synthesis on the disc was linear for a longer period compared to a nonlysogen (Fig. 1); a 30–45% increase in incorporation was usually observed at 30 min. Typically, 100 pmol of DNA were synthesized *in vitro* for 30 min by 10⁸ induced cells. This is ~5–10% of the *in vivo* rate.

ATP is the only ribonucleoside triphosphate required for Mu-specific replication. No difference was observed when all four NTPs were included in the reaction mix. Chloramphenicol added directly to the *in vitro* reaction mixture also had no effect on replication of Mu under these conditions (results not shown).

Since Mu does not shut off host replication, viral DNA must be distinguished from host. Hybridization was used to measure Mu sequences. The DNA to be assayed was purified, denatured, and mixed with a large excess of Mu viral DNA. DNA reassociation was assayed by S-1 nuclease resistance. The kinetics of renaturation of purified Mu viral DNA under these conditions is depicted in Fig. 2. After 60 min of hybridization, 90% of Mu DNA is in a form resistant to S-1 nuclease. Since Mu viral DNA contains approximately 10% random terminal host sequences or inverted G-loops, the reassociation of viral sequences was complete.

To monitor Mu DNA synthesis *in vivo* and *in vitro*, cells pulsed with [³H]thymidine 5 min prior to harvest were lysed on cellophane discs. DNA *in vitro* was labeled using [α -³²P] dGTP. This permits a direct comparison of the amounts of Mu-specific synthesis *in vivo* and *in vitro*. Reassociation kinetics for HB101 DNA (isolated from discs after 15 min of incubation) hybridized to Mu viral DNA is shown in Fig. 2A. Ten per cent of the DNA made *in vivo* or *in vitro* was S-1 resistant after an hour of renaturation. This was unrelated to

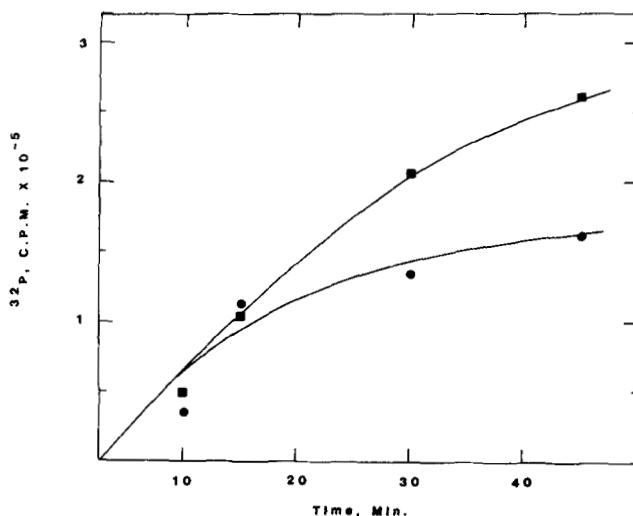


FIG. 1. Kinetics of *in vitro* DNA replication. DNA synthesis was measured at 30 °C for the indicated times using cellophane disc lysates of HB101 (●) and HB101::Mucts (■). The ³²P counts have been normalized to the average amount of *in vivo* ³H label on all discs.

Mu since substitution of unlabeled calf thymus DNA for unlabeled Mu DNA during renaturation gave identical results. A parallel culture of HB101::Mucts had a different profile (Fig. 2B). Fifty nine per cent of the ³H label and 58% of the ³²P label resisted S-1 digestion after an hour of renaturation. Subtracting the 10% background of host reassociation, approximately one-half of the synthesis, both *in vivo* and *in vitro*, was Mu DNA synthesis. In four independent experiments, the percentage of Mu synthesized varied from 30–50% using this experimental protocol.

Semidiscontinuous Replication—The products of *in vitro* replication were analyzed using an alkaline sucrose gradient. Disc reaction mixtures contained either NMN to inhibit *E. coli* ligase or NAD to stimulate ligation. Profiles for the induced, lysogenic strain revealed a pattern of semidiscontinuous replication (Fig. 3A). In the presence of NMN, two discrete size classes of DNA were synthesized in equal amounts, Okazaki pieces and very large fragments. Substitution of NAD for NMN resulted in virtually all of the material sedimenting to the bottom of the gradient (Fig. 3B). Under both of these conditions, the DNA made *in vivo* was large. Profiles for the parental nonlysogenic strain, HB101, were identical with those for HB101::Mucts (not shown).

Both the Okazaki pieces and large [³²P]DNA made *in vitro* were isolated from a sucrose gradient, each hybridized to Mu, and then treated with S-1 nuclease (Table I). Approximately 40% of the Okazaki piece mixture and the large DNA corresponded to Mu sequences in two separate experiments. Therefore, Mu replication *in vitro* must be semidiscontinuous.

Hybridization of Mu DNA Made *In Vitro* to Separated Strands of Mu—The strand specificity of the Mu Okazaki pieces, as well as of the large DNA synthesized in this system, was investigated by hybridization to separated strands of Mu DNA. Mu DNA strands were isolated using their differential density when hybridized to poly(UG) (15). [³²P]DNA made *in vitro* sedimenting with the Okazaki piece peak was isolated from an alkaline sucrose gradient and hybridized to the light and heavy strands of Mu and Mu-specific sequences monitored by digestion with S-1 nuclease (Table I). Preferential hybridization to the light strand was observed. When the large DNA from the sucrose gradient was isolated and hybridized, the reciprocal asymmetric result was obtained, i.e. preferential hybridization to the separated Mu heavy strand. [³H]DNA

² N. P. Higgins, unpublished results.

³ R. Sternglanz, personal communication.

⁴ M. Pato, personal communication.

FIG. 2. S-1 nuclease assay for Mu DNA. Cultures of HB101 and HB101::Mucts grown at 42 °C for 25 min were pulsed with ^3H *in vivo*, and DNA was synthesized *in vitro* at 30 °C for 15 min. DNA isolated from discs or from ^{32}P -labeled Mu virus was denatured together with unlabeled Mu viral DNA and reannealed at 65 °C for the indicated times. The per cent of total radiolabeled DNA which resisted S-1 nuclease digestion is plotted. A shows the ^{32}P label from Mu virus (Δ) or HB101 (\blacksquare) and ^3H label from HB101 (\bullet). B shows ^{32}P label (\blacksquare) and ^3H label (\bullet) from HB101::Mucts.

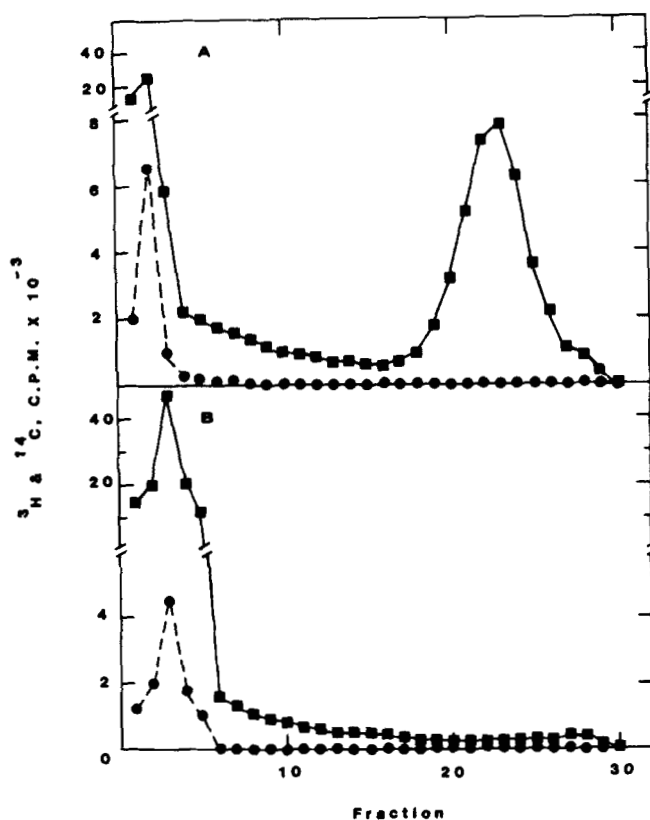
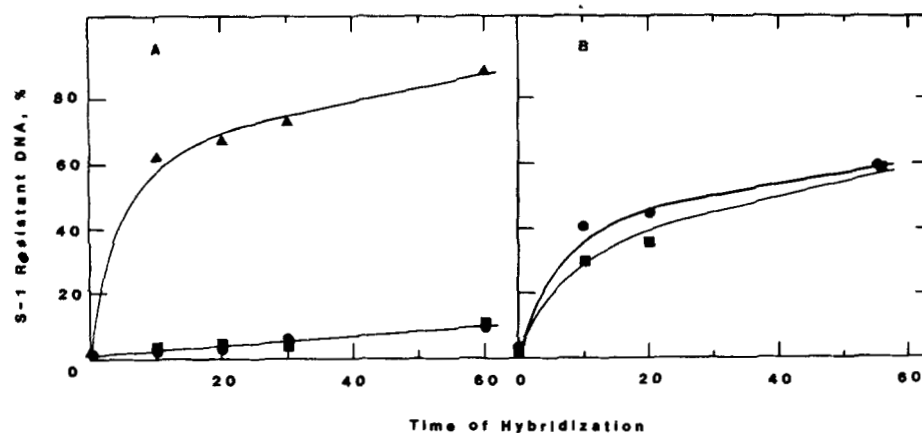


FIG. 3. Alkaline sucrose gradient analysis of DNA from HB101::Mucts cells. An exponential culture of HB101::Mucts grown at 42 °C for 25 min was labeled *in vivo* for 5 min with [^{14}C]thymidine. Incubations were carried out for 15 min on cellophane discs containing either a regular reaction mix with [^3H]dTTP (A) or a mix containing [^3H]dTTP and NAD replacing NMN (B). The disc products were layered onto alkaline sucrose gradients and centrifuged (12). Fractions collected from the bottom of the gradient tubes were precipitated on GFC filters and counted to determine amounts of ^3H (\blacksquare) and ^{14}C (\bullet).

made *in vivo* hybridized symmetrically to the light and heavy Mu strands. Four separate experiments are shown in Table I. These results indicate that the light and heavy strands serve as preferential templates for the discontinuously and continuously synthesized DNA, respectively.

Hybridization of Host Sequences—The asymmetric nature of Mu replication leads to a question of how Mu DNA is linked to the host chromosomal DNA. The template viral strand for continuous synthesis could be preferentially linked to either the continuous or discontinuous template strand of the bacterial chromosome, or the Mu sequences could be

TABLE I

Strand specificity of Mu DNA synthesized in vitro

Cultures of HB101::Mucts thermoinduced at 42 °C for 30 min were spread on cellophane discs and DNA was made *in vitro* for 15 min. Products were sedimented through alkaline sucrose gradients (Fig. 3) and the Okazaki piece peak, or the "continuous" DNA, was pooled and hybridized to indicated strands of Mu viral DNA for 3 h. After S-1 nuclease digestion, the acid-precipitable radioactivity was measured. Data are given for 4 separate experiments and the per cent hybridization (data in parentheses) to indicated strands was calculated after subtracting the blank.

Source of DNA	Hybridization to probe DNA					
	Mu heavy strands		Mu light strands		Mu DNA	None
	<i>cpm</i>	%	<i>cpm</i>	%	%	<i>cpm</i>
Experiment 1						
Okazaki pieces	527	(9.2)	1,556	(32)	1,961 (42)	120
Large pieces ³² P	524	(35)	191	(8.6)	561 (49)	113
Large pieces (<i>in vivo</i> ³ H label)	357	(23)	351	(28)	530 (49)	127
Experiment 2						
Okazaki pieces	753	(10)	2,343	(42)	2,210 (40)	185
Continuous strand	520	(35)	172	(4.7)	534 (36)	118
Experiment 3						
Okazaki pieces	2,885	(13)	9,059	(54)	9,202 (53)	654
Experiment 4						
Okazaki pieces	2,272	(8.6)	7,412	(39)	10,000 (48)	844

randomly oriented with respect to the host strands. In order to examine this question, we hybridized host sequences attached to the light strand of Mu (packaged Mu viral DNA contains 2000–3000 base pairs of host DNA at the right end) to radioactively labeled host Okazaki pieces isolated from uninfected cells. The hybridization of host Okazaki pieces to Mu light and heavy strands had to be carried out for an extended period to allow sufficient time for annealing of the more complex host sequences. Although Mu Okazaki pieces hybridized preferentially to the light strand, host Okazaki pieces showed no strand bias (Table II). Large DNA from uninfected host cells was also annealed to the Mu light and heavy strands, and a similar symmetrical hybridization was observed. Therefore, host DNA which is discontinuously synthesized is associated equally with the light and heavy strand of Mu. This result is consistent with Mu sequences being randomly oriented with respect to the host strands. An alternative, less likely possibility (see "Discussion"), is that Mu has a single fixed orientation throughout the chromosome; the host continuously synthesized strand would be linked to the Mu continuously synthesized strand for one-half of the chromosome, but to the Mu discontinuously synthesized

TABLE II

Strand specificity of host DNA at the ends of Mu viral DNA

A culture of HB101 was incubated at 42 °C for 30 min and replication on a cellophane disc was carried out for 15 min. The DNA was sedimented through an alkaline sucrose gradient and the pooled fractions from the Okazaki piece peak were hybridized to separated strands of Mu for the indicated times. Okazaki pieces in the 23-h experiment were hybridized at twice the concentration of the 3-h and 12-h time points. In Experiment 2, the "middle pieces" represent fractions 12–17 on an alkaline sucrose gradient similar to Fig. 3. This region of gradients from Mu-infected cells hybridizes mainly to the heavy strandlike large pieces. However, it is substantially purified from the excess of host DNA in the large DNA pool so that background in the absence of added light or heavy strands is low. After S-1 nuclease digestion, the acid-precipitable radioactivity was determined.

	Time of hybridization	Hybridization to probe DNA				
		Mu heavy strands		Mu light strands		None
		<i>cpm</i>	%	<i>cpm</i>	%	
Experiment 1						
Okazaki pieces	3 h	212	(2.1)	330	(2.3)	175
	12 h	1300	(8.9)	1290	(8.9)	259
	23 h	3062	(30.3)	3792	(37.5)	337
Experiment 2						
Okazaki pieces	24 h	567	(4.6)	680	(5.5)	175
"Middle" pieces	24 h	1128	(5.4)	1003	(5.6)	395

strand for the other half of the bidirectional host fork. With either possibility, we can conclude that host sequences in packaged virions are not synthesized as part of the same replication event as the Mu sequences.

In the experiments included in Table II, over one-third of host DNA could be annealed to Mu. If the hybridization time is extended to 80 h, over 80% of host sequences become annealed to the Mu viral DNA at the high DNA concentration. This indicates that the majority of and probably all *E. coli* DNA sequences can be attached to Mu DNA and packaged into Mu phages.

DISCUSSION

Bacteriophage Mu replication produces the highest transposition frequency known to occur in any biological system, 100 events/cell in 1 h. By combining a DNA/DNA reassociation assay for detecting Mu sequences with the film lysate technique on cellophane discs, we have demonstrated efficient Mu DNA replication *in vitro*. The *in vitro* system has the potential for analyzing strand asymmetry during replication since it is possible to separate the nascent complementary strands. Any asymmetry detected in one nascent strand should be reflected by a complementary asymmetry in the other strand. Mu replication is particularly favorable for such an analysis.

Mu DNA replication is semidiscontinuous *in vitro*. In an induced Mu lysogen where 40% of the total *in vitro* synthesis was Mu DNA, sealing was completely inhibited by NMN (Fig. 3). One-half of the viral DNA was synthesized as Okazaki pieces and the other half was large pieces. It has been suggested that the phage induces a Mu ligase, coded for by a Mu gene designated *lig* (17). If an ATP-requiring phage ligase were induced, the sedimentation pattern in the presence of NMN would not have been obtained (Fig. 3). Thus, the operative ligase is NAD-requiring; at present, there is no precedent for a NAD-requiring viral induced ligase.

Two apparently conflicting results have been reported on the direction of Mu replication *in vivo*. Using electron microscopy, Harshey *et al.* (18) analyzed structures isolated from cells replicating mini-Mu plasmids. They concluded that mini-Mu replication proceeded from either the left or the right end

with approximately equal frequency. On the basis of asymmetric hybridization of *in vivo* Okazaki pieces to heavy and light strands of Mu, Goosen (19) suggested that most replication proceeded from the left end of Mu rightward (also see Wijffelman and van de Putte (20)). Our results demonstrate that Mu replication occurs predominantly from left to right *in vitro*. In four experiments, an average of 80% of the Mu Okazaki pieces hybridized selectively to the light strand of Mu. An equally strong bias existed for the large pieces; 80% hybridized to the Mu heavy strand (Table I). We conclude that the vast majority of molecules are replicated from left to right across the Mu genome. The data lead to minimum estimates because the viral DNA used to drive hybridization was obtained from phage of an induced lysogen which contains the G inversion segment in both orientations (21). The G segment represents approximately 9% of Mu DNA. We are currently investigating whether Mu Okazaki pieces which hybridize to the heavy strand are accounted for solely by hybridization to the G segment. One alternative possibility is that a small proportion of Mu sequences are being "passively" replicated by the host and, hence, appear to initiate at the right end and proceed leftward.

Our *in vitro* data, and a variety of *in vivo* data, indicate that Mu DNA replication is remarkably similar to host chromosomal synthesis in many respects. Mu not only requires many host replication proteins, but the size distribution of nascent Mu DNA is similar to that of host DNA. Even when ligation is blocked by NMN, the Mu continuous strand can be very large. There is no evidence for a significant accumulation of Mu-sized pieces. The *in vivo* experiments of Pato and Waggoner (22) show that replicating Mu DNA is constantly associated with the host chromosome. We suggest that the reason Mu has many features of host replication is because the semidiscontinuous bacterial replication apparatus is involved.

These experiments indicate that the strand asymmetry of hybridization for Mu sequences does not extend to hybridization of host sequences which occur in the viral DNA. Okazaki pieces isolated from a nonlysogen hybridize equally well to both Mu strands; this is consistent with Mu sequences oriented randomly with respect to the host strands. The random orientation of Mu is also supported by genetic data (23). The strong hybridization asymmetry of the Mu sequences and the hybridization symmetry of their attached host sequences limits the number of tenable replication-transposition models. If unperturbed host replisomes used Mu sequences that were randomly oriented as template strands, no Mu strand asymmetry should have been observed. The Mu asymmetry indicates that phage proteins direct a replisome to go from left to right within Mu sequences.

The establishment of an *in vitro* system for Mu replication opens up the possibility of identifying and isolating intermediates in the transposition cycle. By dissecting Mu DNA synthesis *in vitro*, we are optimistic that the activity of viral proteins necessary for transposition can be characterized.

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